Part II

# Increase of Certain Soluble Liver Proteins Associated with Dye Binding during Aminoazo Dye Hepatocarcinogenesis in the Rat

by

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The second phase (12) of our work of the past year has dealt with the behavior of the proteins of one, small sub-class of these same soluble rat liver h-proteins, the slow-$h_2$ proteins. These proteins represent only 1.0 to 1.9% of all soluble rat liver proteins, exact quantity depending upon experimental conditions to be described. In other terms, the slow-$h_2$ proteins represent only between 0.5 to 1.0% of all proteins of rat liver. Evidences appear to be compatible with the possible direct involvement of this small group of proteins, the "slow-$h_2$" proteins, in azo dye carcinogenesis.

The azo carcinogenic, azo non-carcinogenic, and control diets were fed to rats exactly as described in Part I of this Progress Report. The soluble proteins of liver were then prepared and analyzed electrophoretically by methods also summarized there.

The soluble azoproteins absorb liver maximally at wavelength 405 mμ. Hence, this property may be used to determine the location of azoproteins of separated components in the electrophoresis cell. Figure III shows the absence of light absorption of wavelength greater than 530 mμ by the h-proteins of rats fed DAB or control diets. However, using light of wavelength 390 to 450 mμ, the separated azoproteins from DAB fed rats absorb the light, in contrast to the control diet pattern, shown in juxtaposition. The beginning of the light absorption corresponds to the beginning of the slow-$h_2$ proteins. The lack of absorption by the $h_3$-proteins appears to indicate the relative lack of azoproteins with this component. Similar experiment has been done with the other tested dyes. These experiments do not offer information about the possible azoprotein content of the other adjacent components, since the slow-$h_2$ proteins are present throughout the remainder of the cell. In any event, the slow-$h_2$ proteins are thus shown to contain azoproteins. By direct analyses of isolated fractions in the former study (6), the slow-$h_2$ proteins account for 24% of all the soluble protein-bound dyes of rats fed the more potent carcinogen, 3'-Me-DAB, for $2\frac{1}{2}$ to 3 weeks. Similarly, in the case of rats fed the parent carcinogen, DAB, for $4\frac{1}{2}$ to 5 weeks, the slow-$h_2$ proteins contain 22% of all of the soluble protein-bound dyes; while the corresponding value for the extremely weak carcinogen, 4'-Me-DAB, fed 11 weeks is 24%. Likewise, in the case of the rats fed the non-carcinogen, 2-Me-DAB, for 11 weeks, the slow-$h_2$ proteins contain 19% of all soluble protein-bound dyes.

These slow-$h_2$ proteins, which contain azoproteins, have also been observed to exhibit an interesting response to azo dye feeding. Figure IV illustrates the effect on the amount of slow-$h_2$ proteins caused by the feeding of 3'-Me-DAB for 18-21 days, when the resultant protein-bound dyes are at their maximum level in liver. The average increase in the slow-$h_2$ proteins from such 3'-Me-DAB fed rats has been 48%, compared to the corresponding control proteins.

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In order to determine the cause of this observed increase of the slow-$h_2$ proteins, a series of experiments have been performed the results of which are summarized below. Compared to the 48% increase for the twice as potent carcinogen, 3'-Me-DAB, the parent carcinogen tested, DAB, caused an increase of 60% after feeding $4\frac{1}{2}$ to 5 weeks, its corresponding peak time of liver protein-bound dyes. Similarly, the very weak carcinogen, 4'-Me-DAB, caused a 53% increase of slow-$h_2$ proteins after a feeding of 11 weeks. However, the non-carcinogen, 2-Me-DAB, compared to its control, also fed for the binding peak time (11 weeks), thus far has caused no increase in slow-$h_2$ proteins. This is so despite the fact that 2-Me-DAB is structurally so similar to the azo carcinogens that it is capable of forming relatively large amounts of hepatic protein-bound dyes (3), including those bound to slow-$h_2$ proteins.

That this increase in the amount of slow-$h_2$ proteins is not due to the known stimulated growth, per se, associated with carcinogenic azo dye feeding is seen in the control-like level of these proteins from the 3 days' regenerated rat liver. Likewise, this increase cannot be caused by the decreased dietary intake of azo dye feeding, since the 2-Me-DAB fed rats, which eat less than those on the other tested dyes, did not show any increase of slow-$h_2$ proteins. Similarly, this increase cannot be due to protein binding only, because the 2-Me-DAB causes relatively large amounts of liver protein-bound dyes, including those bound to slow-$h_2$ proteins. Up to the present time, at least, the only reasonable correlation with the increase of the slow-$h_2$ proteins appears to be the necessity for both protein-binding and carcinogenicity. As a purely speculative working hypothesis, which we of course are not prepared to defend, we wonder whether the observed preneoplastic increase of the slow-$h_2$ proteins may constitute a specific response against the effects of their binding of carcinogenic azo dye derivatives, as opposed to those from non-carcinogenic azo dyes. If so, this response of these proteins may be an ultimately unsuccessful attempt to compensate against their dye binding, with the consequent already demonstrated loss of h-proteins in the azo dye induced tumor (5). Therefore, we further wonder whether possibly the slow-$h_2$ proteins may be the specific target liver proteins in azo dye hepatocarcinogenesis.

In summary, a small sub-class of h-proteins, the slow-$h_2$ proteins, have been shown to contain a relatively large share of soluble azoproteins resulting from ingested carcinogenic or non-carcinogenic azo dyes. Only the tested azo carcinogens, DAB, 3'-Me-DAB, and 4'-Me-DAB, induce a relatively large increase in the amount of slow-$h_2$ proteins. The structurally-close non-carcinogen, 2-Me-DAB, although capable of also forming dyes bound to these slow-$h_2$ proteins, has thus far not been found to cause such an increase in these slow-$h_2$ proteins. Thus far, the only apparent correlation with this increase appears to be the necessity for both protein-binding to these slow-$h_2$ proteins and carcinogenicity of ingested azo dyes.

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## References

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Table I

| Diet | protein equivalent color-developed, mg. | γ RNA-P / mg.N | RNA / protein % | γ DNA-P / mg.N | DNA / protein % |
|---|---|---|---|---|---|
| 3'-Me-DAB | 15.5 | 0.2 | 0.3 | undetectable | |
| 3'-Me-DAB | 16.2 | undetectable | | ——— | ——— |
| 3'-Me-DAB | 17.3 | 0.2 | 0.03 | 0.9 | 0.14 |
| avg. | | 0.1 | 0.02 | 0.5 | 0.07 |
| Control | 19.8 | 0.1 | 0.02 | 0.5 | 0.08 |
| Control | 15.8 | 0.1 | 0.02 | 0.4 | 0.06 |
| avg. | | 0.1 | 0.02 | 0.5 | 0.07 |

Nucleic acid analyses of isolated "$h_3$ + slow-$h_2$ + middle $h_2$" fraction from rats fed 3'-Me-DAB or control diets for 18-21 days.

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Table II

| Diet | Wt. of protein mg. | Riboflavin Content γ/gm. | "Flavoprotein" Content* % |
|---|---|---|---|
| 3'-Me-DAB | 171 | < 10 | < 0.15 |
| 3'-ME-DAB | 233 | < 5 | |
| 3'-Me-DAB Control | 348 | 1 | 0.02 |
| 3'-Me-DAB Control | 245 | ca. 0.7 | |

* Based on average 1 mole of flavin per 70,000 grams of protein (11).

Riboflavin and "flavoprotein" contents of dialyzed azo- or control "$h_3$ + slow $h_2$ + middle $h_2$" protein fraction from rats fed 3'-Me-DAB or control diets, respectively, for 18-21 days.

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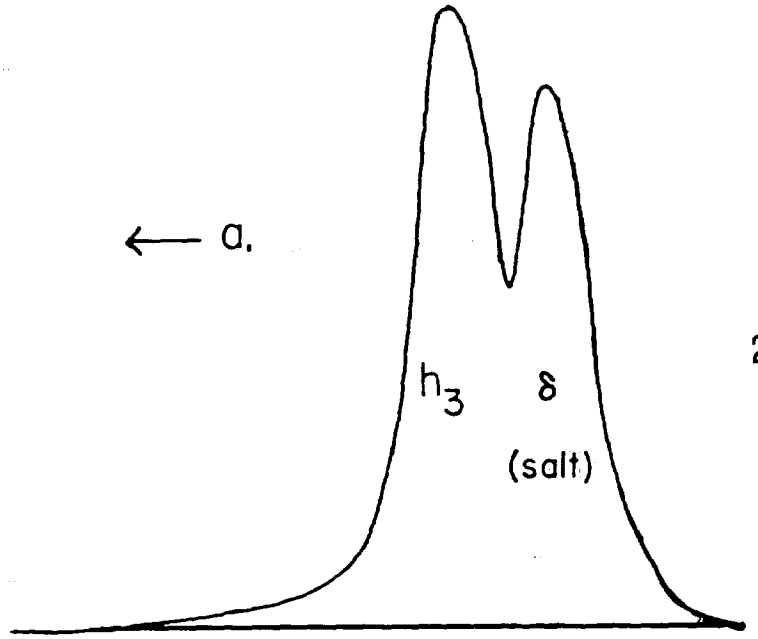
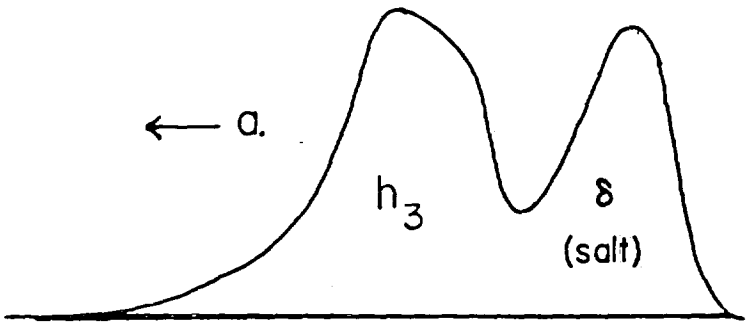
Figure I.




230 mins.
6.52 v./cm.




495 mins.
6.52 v./cm.

# ELECTROPHORETIC DIAGRAMS OF ISOLATED $h_3$ PROTEINS.

3'-Me-DAB CONTROL - 3 WKS.; NaV, 0.1 μ, pH 8.6;
1.3 % PROTEIN

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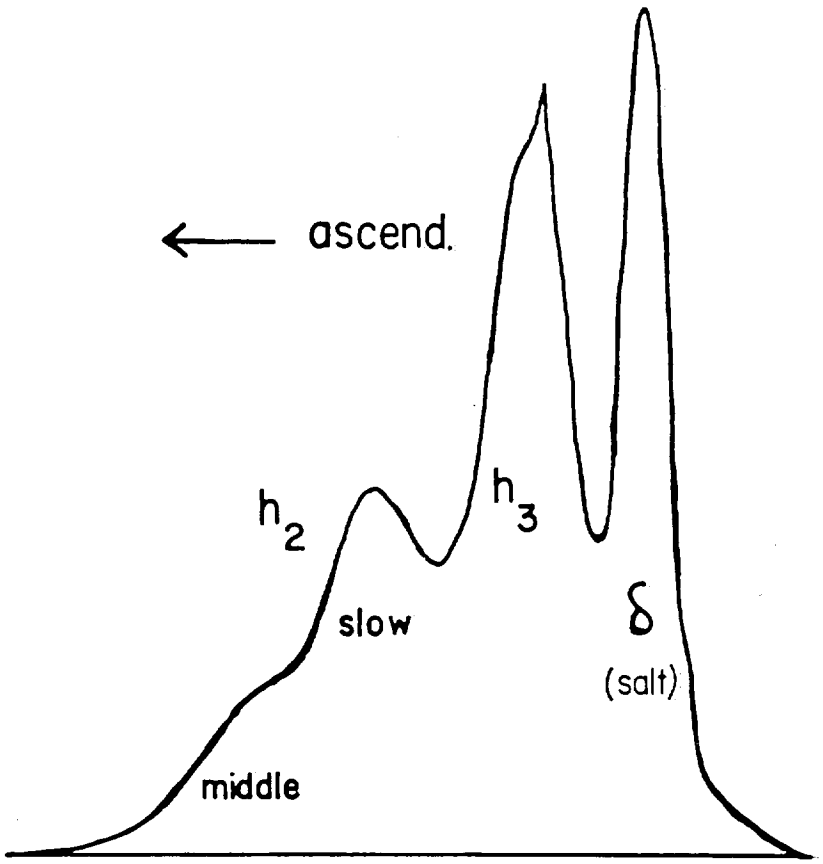
Figure 11.




ELECTROPHORETIC DIAGRAM OF ISOLATED $h_2$ & $h_3$ PROTEINS.

DAB CONTROL-4½ WKS.; 3.1% PROTEIN; NaV, 0.1μ, pH 8.6

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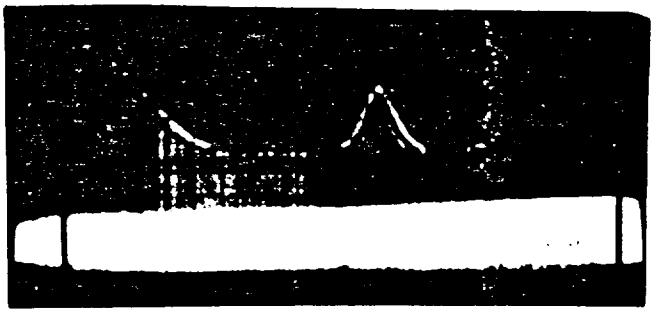
DAB

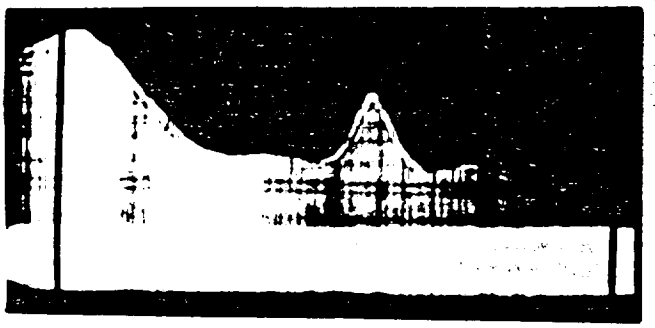
> 530 mμ

CONTROL



> 530 mμ

420 (390-450) mμ



420 (390-450) mμ

SPECTRAL EVIDENCE FOR PRESENCE OF AZO-PROTEINS AMONG SLOW-$h_2$ PROTEINS OF RATS FED 0.06% DAB FOR $4\frac{1}{2}$ WEEKS.

(5190 mins. at 4.30 v./cm. in 0.02 μ NaV + 0.03 μ NaCl, pH 8.6)

Figure III.

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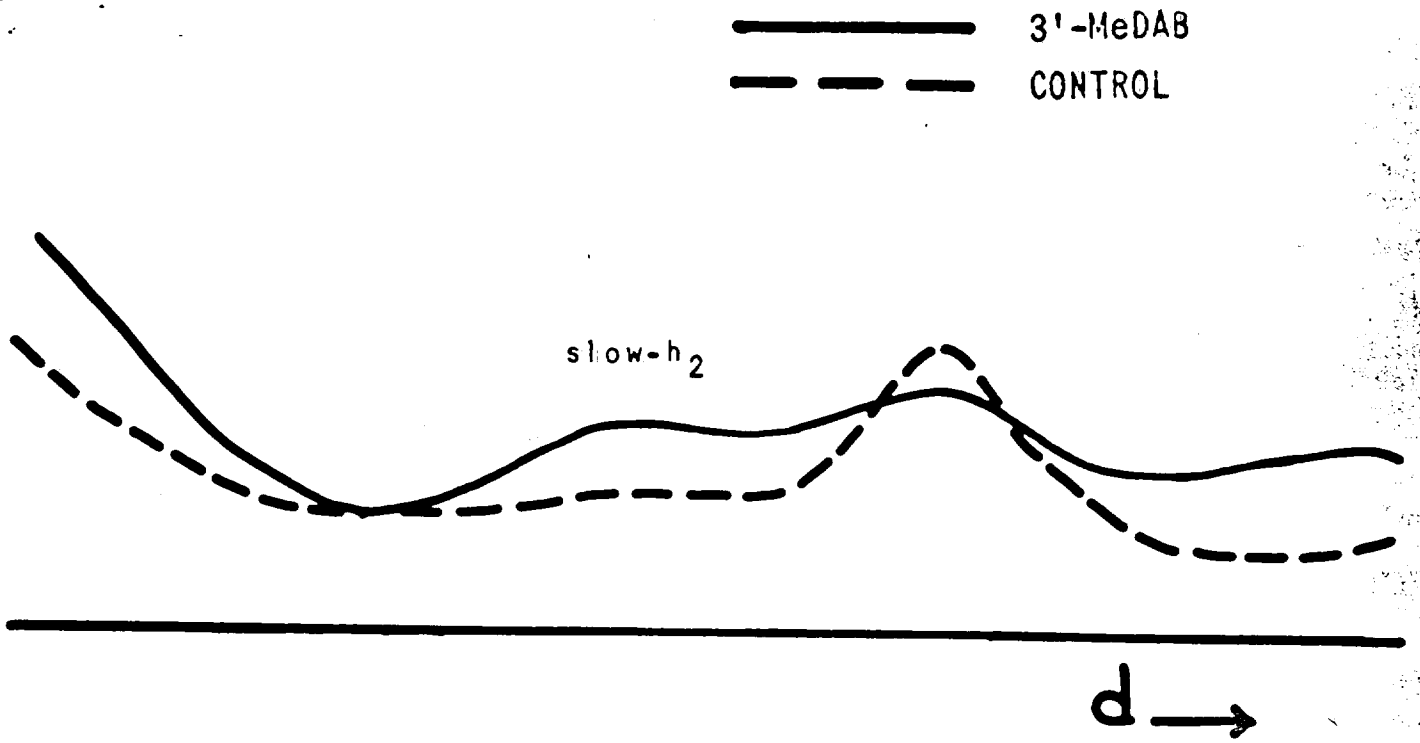


EFFECT ON SLOW-$h_2$ PROTEINS INDUCED BY FEEDING
0.057% 3'-MeDAB FOR 18-21 DAYS

(7150 mins. at 4.30 v./cm. in 0.02μ NaV + 0.03μ NaCl, pH 8.6: 11.4% protein)

Figure IV.

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